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ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

Under the above caption will be published from time to time comments, criticisms and suggestions on technical procedures; minor contributions such as laboratory aids and short cuts which are not considered sufficiently important to warrant a formal paper; and queries.

Obviously comments and criticisms should be signed; queries should be signed but names will be withheld on request; full credit will be given those who contribute laboratory aids, short cuts and the like.

An attempt will be made to obtain answers from authoritative sources to the queries submitted. It must be emphasized that the views expressed in this department are not the opinions of any official body.

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ERRATA

In the article by Giordano and Prestrad (May, 1938 issue of the Supplement), "Improved Method for the Photelometric Determination of Serum Bilirubin" under Reagents, the following change should be made:

1. *Stock solution of pure bilirubin.* Weigh 10 mgm. of pure bilirubin accurately and dissolve in 100 cc. of chloroform.

3. *Solution No. 2.* Dilute 20 cc. of stock solution to 100 cc. with 95 per cent ethyl alcohol.

A SIMPLIFIED METHOD FOR THE DETERMINATION OF BLOOD UREA*

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Such a multitude of methods for the determination of blood urea have been published, that one regrets proposing anything in addition. However, the very multiplicity of the number of procedures in use brings out the fact that the ultimate in desirability has yet not been attained. This supposition seems to be verified by a consideration of the available methods. The aeration methods of Van Slyke[1] and the similar method of Folin and Wu[2]—especially where titration is employed—are both accurate and satisfactory; however, the time required and the chemical skill necessary limit their usefulness in many clinical laboratories. Shorter methods using direct nesslerization of the tungstic acid filtrate have been outlined by Karr[3], Looney[4] and many others. These procedures are quite short, but are not entirely satisfactory. Karr treated the urease filtrate with Nessler's solution directly, but Looney has pointed out that the addition of a colloidal substance (as gum ghatti) is required to prevent precipitation of the colored substance—at least in many instances. This has been our experience in using these procedures. Even though the gum prevents precipitation, it renders the Nesslerized product hazy and prevents accurate color matching; consequently the values obtained are not as reliable as those obtained by the more accurate (but longer) procedures.

Several other types of procedures have been used (notably the manometric urease[5], the xanthydrol[6], the hypobromite[7] the autoclave hydrolysis[2] and the mercuric titration[8] methods), but all have certain objectionable features as to accuracy, technical

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skill or time consumed that make them unsuitable to many laboratories. The entire subject has been carefully reviewed by Seljeskog and Cavett[9].

By the use of a glycerin urease extract, a phosphate buffer, and a Nessler's solution containing a minimum amount of alkali, we have found that direct nesslerization may be carried out without clouding, even though a protective colloid is not used. In fact, the colored solutions remain transparent for 2 hours or more after their preparation.

In preventing this precipitation, all three factors (type of urease, buffer, and amount of base in the reagent) are important. The glycerin urease is definitely superior to even the crystallin material, and all other preparations tried (water solutions, papers, tablets, etc.) were entirely unsatisfactory. The citrate buffer could not be substituted for the phosphate; also the use of Nessler's solutions containing more alkali was accompanied by more tendency for precipitation. In the method presented below we have described the use of these reagents in what we feel is the most satisfactory manner. We have not had an unknown solution precipitate while using this procedure.

METHOD

The proteins are precipitated by the usual tungstic acid procedure. To a large glass test tube graduated or marked at 25 cc.* transfer 5 cc. of the 1:10 blood filtrate, and similarly add 5 cc. of standard nitrogen solution† to a second

* Any type of glass test tube marked at 25 cc. with a file will suffice. We have found 19 x 170 mm. tubes quite satisfactory.

† Prepare a stock standard by dissolving exactly 0.7075 gm. of pure ammonium sulfate in water and making the volume up to 1000 cc. in a volumetric flask. Prepare a working standard by diluting 10 cc. of this solution to 100 cc. with water. 5 cc. = 0.075 mgm. of ammonia nitrogen (equivalent to 15 mgm. of urea N in the method). The dilute solution keeps quite well.

We have found it desirable to use a series of standards in the method, as the values obtained are more accurate and dilution of the nesslerized unknown is unnecessary (except with very high values). The changes in the method are as follows. Prepare a working standard (5 times as strong as the above) by diluting 50 cc. of the stock with water to 100 cc. 1 cc. of this solution = 0.075 mgm. of ammonia nitrogen (equivalent to 15 mgm. of urea N in the method).

Now prepare 5 standard solutions (instead of one as described in the method),

graduated tube. To each of the two test tubes add 2 drops of glycerin urease extract‡ and 4 drops of phosphate buffer solution§. Incubate for 15 minutes in a water bath at 45° to 50°C. Cool by immersing in water (or allowing to stand) and add about 15 cc. of distilled water. Now add 3 cc. of diluted Nessler's solution¶, dilute to the 25 cc. mark, and mix well by inversion. Compare colors in a colorimeter as soon as conveniently possible.

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by using 1, 2, 3, 4, and 5 cc. of the new working standard and adding water to make approximately 5 cc. of total volume. Treat the standards with urease and buffer (as in the method) and nesslerize at the same time as the unknown. In the comparison, the unknown is matched against the appropriate standard. In the calculation use the appropriate value instead of 15. These five standards cover a range of 5 to 100 mgm. per cent. of urea nitrogen. Of course, if the urea value is approximately known, only one standard need be prepared. If desired, a standard of urea can be used instead of the ammonium salt. It is prepared by dissolving 0.0643 gm. of pure urea in 2000 cc. of water. 5 cc. = 0.075 gm. of ammonia nitrogen. This solution has the disadvantage of not keeping as well as the ammonium salt.

‡ *Glycerin Urease Solution.* This is prepared by a modification of Koch's method (10). Shake 15 gm. of permutit with 200 cc. of 2 per cent acetic acid, decant the aqueous phase and wash two or three times with distilled water. To the damp permutit add 50 cc. of 0.001 normal sulfuric acid and 30 gm. of jack bean meal (Arlington Chemical Co.). Shake gently for 30 minutes, add 150 cc. of glycerol and mix thoroughly. Let stand over night and filter through several thicknesses of gauze. Centrifuge the filtrate until essentially clear (there is an opalescent haziness that cannot be entirely removed). The filtrate is, however, very active and stable. The equivalent of 0.03 to 0.05 cc. will convert 2 mgm. of urea nitrogen completely into ammonia (in a 5 cc. volume) at 45° to 50°C. in fifteen minutes. Two drops are sufficient for most urea determinations. Such an extract will keep its full activity for at least a year at room temperature and exposed to the ordinary room illumination; however, keeping the extract in the icebox is to be preferred if convenient.

§ *Phosphate Buffer.* Transfer 14 gm. of sodium pyrophosphate (U.S.P.) and 2.0 cc. of glacial (85 per cent) phosphoric acid into a 250 cc. volumetric flask and make up to volume with distilled water. The solution keeps indefinitely.

¶ *Nessler's Solution.* (a) Double Iodide. To 75 gm. of KI and 110 gm. of pure iodine in a 250 cc. flask, add 50 cc. of water and 70–75 gm. of metallic mercury. Shake the flask continually and vigorously until the dissolved iodine has nearly all disappeared. The solution becomes quite hot. When the iodine color is visibly pale, cool in running water, but continue to shake until the red color of the iodine has been replaced by the green color of the double iodide. Do not cool too soon! Separate the solution from the excess mercury by de-

The urea nitrogen in the unknown solution is calculated from the following equation:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 15 = \text{mgm. of Urea N per 100 cc. of blood}$$

If a urea nitrogen value of 50 or above is present, the determination is preferably repeated with smaller amounts of filtrate‖. However, even if the value is high the sample can usually be saved by adding one or more volumes of water (containing 3 cc. of diluted Nessler's solution + 2 drops of urease solution + 4 drops of buffer solution per 25 cc. of volume) to the unknown solution, comparing the color with that of the standard, and multiplying the value obtained by the dilution of the unknown.

The final nesslerized extract should be practically clear; if cloudiness appears there has been some break in the technique and the determination is of no value. The test tubes should be cleaned with dilute nitric acid between each set of analyses, as a trace of the mercury salts inhibits the action of the urease.

SUMMARY

1. A direct nesslerization technique has been outlined for the determination of blood urea nitrogen.

2. This procedure is carried out on the tungstic acid filtrate, without the use of gum ghatti to prevent precipitation of the colored material.

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cantation, and wash with liberal amounts of distilled water. Dilute the washings and solution to a volume of 2000 cc. with distilled water.

(b) Working solution for urea determination. Mix 3 parts of the double iodide with 3 parts of water. To this mixture add 14 parts of freshly prepared 5 per cent NaOH. This solution keeps at least several months in brown bottles. (The double iodide solution keeps indefinitely.)

‖ See sub-note 2 to page 154. If five standards are used instead of only one, the dilution of the filtrate with high urea values need not be done unless the value is above 100 mg.

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ANALYSIS OF URINARY CALCULI*

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The procedure described below is the same as that reported previously.[1] Directions for the preparation of reagents, together with notes on technique, and modifications of some of the tests, have been added.

Prior to the analysis proper, it is desirable to note briefly the physical (internal) structure of the specimen. Under this heading are included the following observations:

(1) Stratification, or lack of stratification (uniform composition) of the specimen. By this is meant the occurrence or lack of concentric layers visible to the naked eye. The concentric layers may be firmly attached to each other, or loosely held together.
(2) Nucleus, or the occurrence of an internal, centrally located area which to the naked eye appears separate and distinct from the surrounding mass. The nucleus may

* Received for publication April 21st, 1938.

be "pseudo" or "true." The distinction rests upon the chemical analysis, and is discussed below.

PREPARATION OF STONE FOR ANALYSIS, AND MACROSCOPIC EXAMINATION

The stone is placed on a clean piece of filter paper, moistened with alcohol, and scraped free of adherent blood with a scalpel. The specimen is then cut or sawed in half along its shorter axis, and examined for the presence of nucleus and stratification. When the latter is present, a great many layers may occur. In any event, separate layers necessitate separate analysis, before conclusions are drawn as to their chemical identity. Difference in color between concentric layers, does not necessitate a difference in chemical composition. Color is dependent upon several factors, among which are included the state of aggregation, concentration, the conditions of precipitation, and the amount of organic material present in the form of cell débris; and for such reasons, is chemically inconclusive. Usually the concentric layers can be easily pried loose with a scalpel. In other cases they must be separated by careful scraping. When complete separation between layers cannot be effected, separate analysis is inconclusive. Generally separate layers vary in composition only in a quantitative sense. It is noticed that, usually, where two concentric rings alternate, the separate layers appear to progressively merge as they approach the nucleus or center of the stone.

A nucleus may be chemically identical with the rest of the stone. Such a structure is reported as a "pseudo" nucleus, as distinguished from the chemically distinct or "true" nucleus. The nucleus may also differ from the remainder of the stone in the proportions of identical substances contained. With a little practice, it is possible to make such distinction even with the qualitative scheme described. Lastly, a foreign body may compose the nucleus. As an example of this type, the tip of a broken catheter has been encountered.

Logically, stratification always implies the occurrence of a nucleus, either pseudo or true. A specimen possessing only two distinct areas, is reported as *Calculus, Uniform in Composition, with Nucleus (Pseudo or True)*. Stones which show no internal differentiation are reported as being of *Uniform Composition*. Stratification is always noted, and the chemical identity or difference of the concentric rings reported.

Preparatory to chemical analysis, the stone, or a portion thereof, is ground to a fine powder in a small glass mortar, using a glass pestle. Separate layers, and nucleus, are separately ground.

REAGENTS

Nitric Acid, concentrated.

Hydrochloric Acid, 10 per cent: Dilute 30 cc. of the concentrated acid to 100 cc.

Acetic Acid, 5 per cent: Dilute 5 cc. of the concentrated acid (Glacial) to 100 cc.

Ammonium Hydroxide, 15 per cent: Dilute 55 cc. concentrated Ammonium Hydroxide to 100 cc.

Ammonium Hydroxide, 20 per cent: Dilute 70 cc. concentrated Ammonium Hydroxide to 100 cc.

Potassium Permanganate, 0.01 N.: Dissolve 0.32 gm. solid Potassium Permanganate in water. Dilute to 1 liter.

Sodium Hydroxide, 20 per cent: Dissolve 20 gms. solid Sodium Hydroxide in 100 cc. water.

Ammonium Molybdate Reagent: To 72 gms. Molybdic Anhydride (MoO_3) add 130 cc. water and 75 cc. NH_4OH (15 N.). Stir until practically all of the solid has dissolved. Then add to it slowly and with constant stirring, a solution of 240 cc. Nitric Acid (conc.) and 500 cc. water. Let stand for 2–3 days, filter, and use the clear filtrate. The same agent can be prepared by dissolving 90 gms. of pure Ammonium Molybdate in 100 cc. Ammonium Hydroxide (6 N.), followed by addition of 240 gms. Ammonium Nitrate, and diluting to 1 liter.

Nessler Reagent: As described for use in the determination of Non Protein Nitrogen; Folin, Laboratory Manual of Biological Chemistry.

PROCEDURE

It is desirable to take approximately equal quantities of pulverized sample for each of the individual tests described below. A glass or platinum loop of the ordinary size is an adequate measure of the individual amounts necessary.

Phosphates: Place a small amount of the pulverized sample in a Pyrex tube. Add about 5 cc. ammonium molybdate reagent, and an equal volume of concentrated nitric acid. Carefully heat to boiling. A yellow precipitate indicates phosphates. Phosphates when present can be regarded as a mixture of three types; Calcium, Magnesium, and Magnesium Ammonium (Triple Phosphate).

Carbonate: Place a small amount of pulverized sample on a glass slide. Add 1 drop of HCL (10 per cent). Effervescence indicates carbonate. Report as Calcium Carbonate. Relatively large quantities of calcium carbonate show up equally well during the test for Oxalate, described below.

Oxalate: Add approximately 10 cc. HCL (10 per cent) to a portion of pulverized sample. Effervescence at this point indicates carbonate. Filter the mixture into a small casserole, make alkaline to litmus with ammonium hydroxide (15 per cent, then just acid with acetic acid (5 per cent). Permit 15–30 minutes to elapse before proceeding to the next step. The ammonia precipitates both phosphates and oxalate, while the subsequent acidification with acetic acid dissolves the Phosphates, but not the oxalate. Transfer the acid mixture to a test tube and centrifuge until the precipitate, if any, is firmly packed down at the bottom of the tube. Decant the supernatant solution,

place a drop of the sediment on a clean slide, and examine microscopically under low power for the characteristic crystals of calcium oxalate. A crystalline precipitate in the acetic acid solution, is, in itself, an indication of oxalate.

If the above crystals are not characteristic, combine the remainder of the suspected oxalate sediment, add a few cc. of 1 N sulfuric acid, immerse in a boiling water bath for 1–2 minutes, and add dropwise 0.01 N potassium permanganate. Oxalate readily decolorizes permanganate.

Cystine: Add 5–10 cc. ammonium hydroxide (20 per cent) to a small portion of pulverized sample, shake vigorously for a few seconds, filter, and place a few drops of filtrate on a clean slide. Upon the spontaneous evaporation of ammonia, the characteristic flat hexagonal crystals of cystine can be recognized under low power.

Uric Acid-Urates: Place a small quantity of powdered specimen in a porcelain casserole. Add a few drops of concentrated nitric acid, and evaporate *to dryness* on a water bath. A red or orange color signifies uric acid, or urates, or both. (An orange color is suggestive of calcium urate, while a red color suggests uric acid or ammonium urate.) The color so obtained can be intensified by exposure to ammonia fumes. If the specimen is composed entirely of uric acid or ammonium urate, it will completely volatilize when heated on platinum. The urates of calcium and magnesium, on the other hand, will leave behind a white ash whose aqueous solution is strongly alkaline to litmus. Furthermore, an aqueous solution of ammonium urate, in common with inorganic ammonium salts, reacts to Nessler Reagent, with deepening of color. In order to apply this test to calculi, shake some pulverized specimen with water, filter, and add Nessler Reagent to the filtrate. Compare the color so produced with that obtained in another tube of similar bore, in which water is used in place of filtrate, and Nessler Reagent is added to the same height as in the unknown. If in a given specimen of calculus, large quantities of phosphates and oxalate, occur singly or together in the presence of a positive reaction for uric acid-urates as evidenced by the nitric acid test, it is legitimate to conclude that the uric acid is present primarily in the form of its salts, or urates.

Xanthine: The nitric acid test (described under uric acid-urates) when negative, produces a lemon yellow residue. Add to this a few drops of 20 per cent sodium hydroxide. In the presence of Xanthine, a red color is produced.

Concerning the microscopic examination of crystals for the purpose of identification of specific compounds, one must proceed with caution. Crystalline form is often dependent on the concentration of the test solution, concentration of the precipitating agent, and final dilution. At times it is not diagnostic because of the resemblance of two or more substances when routinely examined for crystalline form. Calcium oxalate is usually recognized with ease, other times must be checked against the medium in which it has precipitated, and its power to reduce permanganate, as described. If phosphates are being examined microscopically, care must be taken not to mistake ammonium chloride crystals for the feathery form of triple phosphate. The former begin to appear

shortly after the slide has begun to dry. It is desirable, however, to examine microscopically a specimen withdrawn from the casserole in which phosphates have been precipitated with ammonia, allowing about five minutes to elapse between alkalization and withdrawal of sample. There is the possibility of identifying here phosphates, oxalate, and urates, and to note the relative amount of organic matter present in the form of cell débris. It is, however, inadvisable to draw conclusions as to the specific type of phosphate originally present in the stone, since the conditions under which the precipitation is made, are not standardized.

SCHEME OF ANALYSIS—SUMMARY

1. Pulverized specimen + Ammonium Molybdate Reagent + HNO_3 (conc.) $\xrightarrow{\Delta}$ yellow ppt. = Phosphates
2. Pulverized specimen + NH_4OH (20 per cent). Filter. Flat hexagonal crystals under low power = Cystine
3. Pulverized specimen + HCl (10 per cent) → Evolution of gas (CO_2) = Calcium Carbonate

   ↓ Filter

   (a) Filtrate made alkaline (NH_4OH – 15 per cent)
   (b) Filtrate made *just* acid (Acetic – 5 per cent)
   (c) Centrifuge. Examine sediment under low power for CaC_2O_4
   (or) Sediment + H_2SO_4 (1N.) + Δ + $KMnO_4$ (0.01N) decolorization of permanganate = Calcium Oxalate
4. Pulverized specimen + HNO_3 (conc.) to $\frac{H_2O\ bath}{to\ dryness}$ Red or Orange color = Uric Acid, Urates
5. Lemon-yellow residue from (4) + NaOH (20 per cent) → Red color = Xanthine

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A SIMPLIFIED PROCEDURE FOR THE DETERMINATION OF MERCURY IN URINE

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In following the urinary excretion of mercury after the therapeutic administration of medicinal mercurials, or after mercury

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poisoning, a method of estimation which combines speed and sensitivity with reasonable accuracy is desirable. A simplification of the extraction-titration procedure of Winkler[1] has been devised to meet these requirements in cases where the concentration of mercury in the urine to be examined is at least 0.05 mgm. in 50 cc. If, on the other hand, the concentration exceeds one mgm. in 50 cc. an appropriate aliquot portion is taken.

SPECIAL REAGENTS REQUIRED

Dithizone: 25 mgm. of diphenylthiocarbazone (Eastman Kodak Co.) are dissolved in a liter of carbon tetrachloride.

Standard mercuric nitrate solution: 0.500 gm. of metallic mercury (Kahlbaum's "for analysis") are dissolved in concentrated nitric acid and made up to 500 cc. To 10.00 cc. of this solution are added 10 cc. of concentrated nitric acid and diluted to exactly one liter. One cubic centimeter of the latter solution contains 0.01 mgm. of mercury.

DESCRIPTION OF METHOD

To 50 cc. of urine in a 500 cc. Kjeldahl flask are added 1 cc. of concentrated sulphuric acid, 5 cc. of concentrated nitric acid and crystals of solid potassium permanganate until a decided violet color remains or brown precipitate forms. A "cold finger" condenser* is hung in the neck of the flask and the mixture refluxed over a microburner until clear. More permanganate is now added as required and the solution refluxed until clearing no longer takes place on boiling 10 to 15 minutes after the last portion has been added. Every time the condenser is removed for the addition of permanganate, care must be taken to avoid loss of any condensate thereon. When the condenser is removed for the last time it is thoroughly rinsed and the rinsings collected into the digestion flask. While the flask and contents are still quite warm, small portions of solid potassium nitrite are added until all manganese dioxide has been decomposed and the solution is clear and colorless. The excess nitrous acid is in turn removed by the addition of about one gram of hydroxylamine sulphate crystals. The solution is then quantitatively rinsed into a 250 cc. separatory funnel, keeping the total volume below 100 cc.

The completely digested urine which is free of oxidizing agents is now extracted with dithizone reagent. The dithizone is placed into a burette and the position of the meniscus recorded. Three cc. of dithizone are added to the material in the separatory funnel, thoroughly shaken, and then allowed to stand. If the carbon tetrachloride layer does not assume a bright orange color (but remains green or brownish orange) the mercury content of the 50 cc. urine

* A 10 x 1 inch pyrex test tube through which water is circulating is convenient for this purpose.

sample is less than 0.03 mg. and a much larger volume of urine should be taken and analyzed by the original Winkler procedure. Should the non-aqueous layer, however, assume a bright orange color, then successive 5 cc. portions of the dithizone solution are added to the material in the separatory funnel. After each addition the separatory funnel is thoroughly shaken and then allowed to stand. Each 5 cc. portion of carbon tetrachloride extract which assumes the bright orange color of the mercury-dithizone complex is discarded. This process of extracting and discarding is continued until a carbon tetrachloride layer assumes an "off color" (green to a brownish shade of orange). This last 5 cc. portion of carbon tetrachloride is not drawn off. A measured amount of the standard mercuric nitrate solution is now added, such that after the separatory funnel has been vigorously shaken, a slight excess of free mercuric nitrate remains in the aqueous layer. This is indicated by the bright orange color of the carbon tetrachloride layer. After this orange colored layer has completely separated, it is drawn off and discarded. The titration is now completed with successive 0.1 cc. portions of the dithizone reagent from the burette. After each addition the contents of the separatory funnel are thoroughly shaken, the carbon tetrachloride layer is allowed to settle and is carefully removed and discarded. The end point is indicated when a 0.1 cc. portion of dithizone assumes the "off color." The total volume of dithizone taken from the burette is recorded.

The dithizone solution must be standardized with each series of determinations since it deteriorates slowly. To 10.0 cc. of the standard mercuric nitrate solution are added one cc. of concentrated sulphuric acid and the volume is made up to about 50 cc. The titration is then conducted exactly as described above. As an illustration the standardization of the dithizone would be calculated as follows:

10.0 cc. standard mercuric nitrate solution taken.

0.3 cc. standard mercuric nitrate solution added near end point in three portions, to get bright orange color of chloroform layer

10.3 x 0.01 = 0.103 mg. mercury; the equivalent of the total volume of dithizone solution required.

11.2 cc. total volume of dithizone used for titration.

0.103/11.2 = 0.0092 mg. mercury; the equivalent of one cc. dithizone solution.

The mercury content of the urine sample is calculated as follows:

(cc. dithizone used) x (Hg equivalent of dithizone)—(cc. standard mercury solution added at end point) x 0.01 = mg. mercury in sample analyzed.

Known amounts of mercury were added to a series of 50 cc. portions of normal urine. The analytical results are given in table 1. Ordinarily chemically pure reagents are satisfactory and the blank was found to be negligible. The time required for a series of five determinations is about three hours. Samples of urine with a concentration of less than 0.05 mg. mercury in 50 cc.

must be subjected to the complete procedure described by Winkler[1] which involves a preliminary concentration by extraction. Results of analyses using the Winkler method on 200 cc. portions of normal urine to which known quantities of mercury were added are given in table 2.

TABLE 1

RESULTS OF ANALYSES BY SIMPLIFIED METHOD

MGM. Hg ADDED	MGM. Hg FOUND	RELATIVE PER CENT ERROR
0.100	0.100	0
0.100	0.101	+1
0.300	0.289	−3
1.000	0.991	−1
1.000	0.986	−2

TABLE 2

RESULTS OF ANALYSES BY WINKLER'S METHOD

MGM. Hg ADDED	MGM. Hg FOUND	RELATIVE PER CENT ERROR
0.051	0.050	−2
0.100	0.101	+1
0.152	0.141	−7
0.163	0.157	−4
0.300	0.312	+4
0.356	0.364	+2
0.509	0.486	−5

A mean blank correction of 0.015 mgm. has been subtracted from each result. The time required for a series of five determinations is about five hours.

SUMMARY

1. A simplified procedure is described for the rapid estimation of small amounts of mercury in urine.

2. Winkler's original method has been applied to urine samples and found to be satisfactory.

3. The simplified procedure is more rapid than the Winkler method and equally accurate.

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Compare: FISCHER, H. AND LEOPOLDI, G.: Z. anal. Chem., **103:** 241, 1935.

HORTEGA'S SILVER IMPREGNATION TECHNIQUE, USES AND APPLICATION*

WM. M. GERMAN

From the Pathological Laboratories of the Good Samaritan Hospital, Cincinnati, Ohio

Introduction: A combination of good fortune, the wanderlust and the courtesy of some good friends put me in the Cancer Institute at Madrid, Spain in the summer of 1935, before the present revolution. My gracious host, Dr. Del Rio Hortega at once put me to work in an intensive course in histological technique, from which I emerged with a much better knowledge of the microscopic anatomy of the central nervous system and a much better judge of its tumors. These silver impregnation methods have been so generally useful and so helpful to me in the classification of neoplasms, especially of the central nervous system, that, at the suggestion of some of the members of the society, I am passing them on to you with comments on their application.

It may be said in general that Hortega's silver technique produces brilliant results. The methods are dependable, and using reagents which are stable the results are consistent. The stains have a high degree of specificity for either tissue cells or cell structures, produce preparations of exceptional contrast for photographic purposes.

I shall divide the methods into two groups, viz.; general stains and specific stains, and shall describe them a few at a time in the succeeding numbers of the TECHNICAL SUPPLEMENT.

GENERAL INSTRUCTIONS

All these methods require fixation in the usual formol solution adjusted to approximate neutrality. All the sections should be cut by the freezing microtome and should be not over 15 microns thick. Very thin sections can be obtained by observing the following precautions:

1. Place a small square of water saturated filter paper on the stage of the freezing microtome.

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* This is the first of a series of three papers dealing with this subject.

Received for publication May 27, 1938.

2. Upon this place the block of tissue to be cut.

3. Build up use of a medicine dropper and water, as the freezing progresses, a small mass of ice around the tissue, so that when freezing is complete (do not over-freeze) the tissue is embedded in a small block of ice similar to tissue embedded in paraffin by the paraffin method. The precautions serve as an insulation to prevent too rapid and over-freezing and too rapid thawing. A little experience will enable one to determine the optimum temperature at which excellent sections may be cut in large numbers.

Materials Required:

1 doz. low form Stender staining dishes, 36 mm. diameter, 10 cc. capacity.
½ doz. low form Stender dishes, 50 mm. diameter, 30 cc. capacity.
½ doz. low form Stender dishes, 60 mm. diameter, 50 cc. capacity.
Tripod and micro burner.
A thin, tapered, slightly angulated glass rod for handling sections.
Dropping bottles for all but stock reagents.
Amber glass stoppered bottles for silver reagents.
Slides, No. 1 cover glasses, size to suit sections.
Coarse filter paper in sheets and cut in suitable size to blot sections.
Xylol free Canada balsam.

HORTEGA GENERAL NUCLEAR STAIN

Reagents required:

1. Silver carbonate solution:
   10 per cent Silver nitrate sol., 5 cc.
   5 per cent C. P. Sodium carbonate sol., 15 cc.
   Ammonium hydroxide conc. drop by drop until precipitate is completely dissolved.
   Distilled water to 75 cc.
2. 0.4 per cent C.P. Gold chloride solution.
3. 5 per cent Sodium hyposulphite solution.
4. Picro-indigo solution:
   1 gram Indigo-carmine.
   200 cc. Dist. water.
   Picric acid sufficient to saturate but no more.
5. Picro-fuchsin solution:
   Acid fuchsin, 0.5 gram.
   Distilled water, 200 cc.
   Picric acid—just sufficient to saturate.
6. Formaldehyde solution 1 per cent.

GENERAL NUCLEAR STAIN

Technique:

1. Sections by frozen technique (very thin).
2. Float in distilled water.

3. Wash twice in distilled water.
4. Place sections in silver carbonate solution at room temperature for 5 to 10 minutes. (15 to 50 seconds for macrophages)
5. Wash quickly in distilled water.
6. Place sections in 1 per cent formol solution for 1 minute.
7. Wash in distilled water for 1 minute.
8. Place sections in 0.4 per cent gold chloride solution for 2 minutes. (Note: This step is optional and minimizes overstaining with silver stain, thus making stroma lighter for use of contrast stain).
9. Wash in distilled water 1 minute.

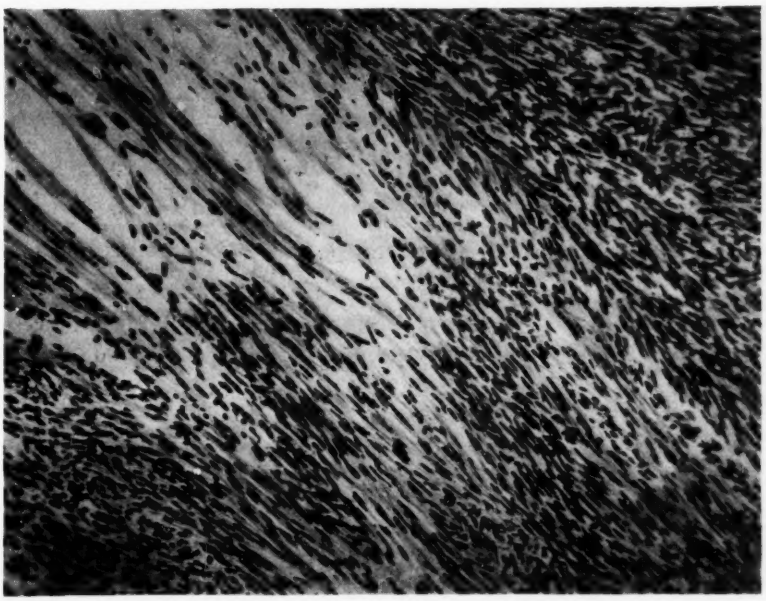

FIG. 1. PHOTOMICROGRAPH SHOWING USE OF GENERAL NUCLEAR STAIN
Picro-indigo used as counterstain

10. Place sections in 5 per cent sod. hyposulphite solution 1 minute.
11. Wash in distilled water.
12. Mount section on clean slide and blot with filter paper.
13. Cover section on slide with complimentary stain, picro-indigo or picro-fuchsin, for 1 minute.
14. Drain off excess stain, blot with filter paper.
15. Dehydrate by flooding slide with absolute alcohol, drain.
16. Blot with filter paper.
17. Clear by flooding slide with pure creosote, drain, blot with paper.
18. Mount in balsam, cover with No. 1 coverglass.

Results: If picro-indigo stain is used, nuclei stain black or grey, cytoplasmic granules the same, mitoses are shown admirably. The detail is sufficient for use of oil immersion lens. Collagenous connective tissue stains green, calcium deposits blue. If procedure 8 is omitted, nuclei stain brown, degenerated collagen receives brown silver stain, cell cytoplasm and muscle cells stain yellowish brown, and collagenous connective tissue stains green. (Figs. 1 and 2.)

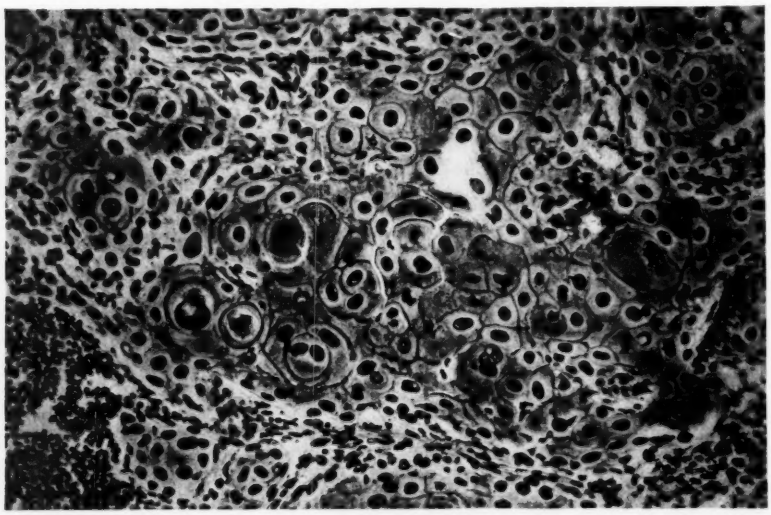

FIG. 2. GENERAL NUCLEAR STAIN. PHOTOMICROGRAPH OF SECTION OF A METASTATIC SQUAMOUS CELL CARCINOMA
Note cell outlines and nuclear details

GENERAL IMPREGNATION METHOD FOR NUCLEI AND CONNECTIVE TISSUE

1. Sections cut by frozen technique (very thin).
2. Wash three times in distilled water.
3. Place sections in 10 cc. silver carbonate solution plus 3 drops Pyridine.
4. Cover with watch crystal the size of staining dish; heat very slowly with micro-burner, 50 to 55 degrees centrigrade, with occasional rotation of dish, until sections assume rich brown color of tobacco.
5. Wash in distilled water.
6. Place sections in 10 per cent formalin ½ minute
7. Wash in distilled water.
8. Divide sections into two groups, 1 and 2.

Group 1:

1. Place sections in 5 per cent sod. hyposulphite 1 minute with agitation.
2. Wash in distilled water.

3. Mount section on clean slide, drain, blot.
4. Dehydrate on slide by flooding with absolute alcohol; drain, blot.
5. Clear by flooding with creosote; drain, blot.
6. Mount in balsam with No. 1 coverglass.

Group 2:

1. Place sections in 0.4 per cent gold chloride solution at room temperature.
2. Heat very slowly (without cover) until very dark.
3. Wash in distilled water.
4. Place in 5 per cent sodium hyposulphite 1 minute with slight agitation.
5. Wash in distilled water.

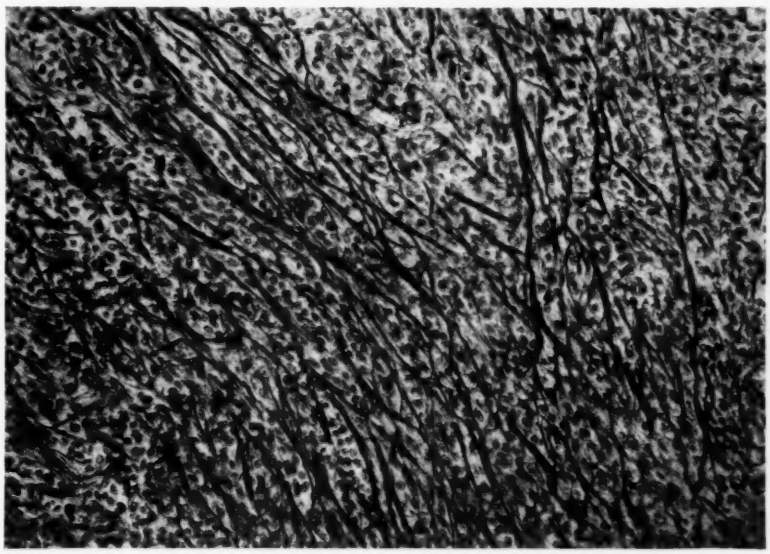

FIG. 3. PHOTOMICROGRAPH SHOWING USE OF THE GENERAL IMPREGNATION METHOD FOR CONNECTIVE TISSUE AND NUCLEI. SECTION OF SCIRRHOUS CARCINOMA OF THE BREAST

6. Mount section on slide, blot, dehydrate, clear with creosote as above; mount in balsam with No. 1 coverglass.

Results: Group 1: Nuclei stain black, protoplasmic granules black, cytoplasm brown, connective tissue fibrile brown to black depending on their character and density and size. (Figure 3.)

Group 2: Nuclei and protoplasmic granules stain black, cytoplasm lavender, connective tissue fibrils mahogany red to black depending on character. Degenerated collagenous connective tissue contains argentophile granules.

HORTEGA STAIN FOR EPITHELIAL FIBRILS, EPITHELIAL BRIDGES AND PROTOPLASMIC RETICULUM

A. Materials: As before suggested.
B. Technique:
1. Fixation in formol.
2. Sections by freezing microtome (very thin).
3. Wash rapidly in distilled water.
4. Place sections in 10 cc. silver carbonate solution plus 10 drops of pyridine.

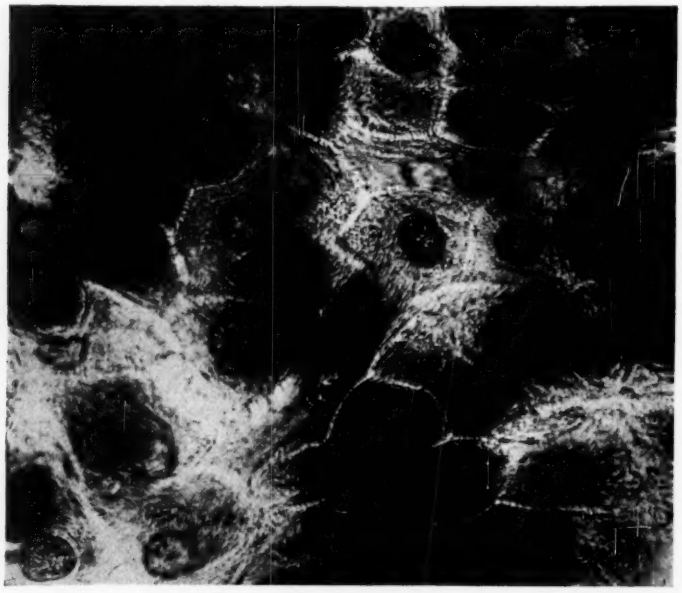

FIG. 4. PHOTOMICROGRAPH OF SECTION OF SQUAMOUS CELL CARCINOMA. OIL IMMERSION

Note epithelial bridges and long cellular fibrils

5. Cover with watch crystal and heat very slowly on micro-burner at 50 to 55 degrees centigrade until rich brown color.
6. Wash twice in distilled water.
7. Divide sections into two groups, 1 and 2.

Group 1.

Place sections in 5 per cent sodium hyposulphite for 1 minute.
Wash sections in distilled water.
Mount section on slide, drain, blot.

Dehydrate by flooding slide with alcohol, drain, blot.
Clear by flooding slide with creosote, drain, blot.
Mount with balsam and No. 1 cover-glass.

Group 2.

Place sections in 0.4 per cent gold chloride solution at room temperature.
Heat, without cover, slowly 50 to 55 degrees until sections are dark.
Wash in distilled water.
Place sections in 5 per cent sodium hyposulphite solution 1 to 2 minutes.
Wash in distilled water.
Mount section on slide, drain, blot.
Dehydrate by flooding with alcohol, drain, blot.
Clear by flooding with creosote, drain, blot.
Mount with balsam and No. 1 cover-glass.

Results: The section may be examined with high dry or oil immersion objective; intercellular bridges and epithelial fibrils may be seen passing from cytoplasm of one cell to that of others. (Fig. 4)

NOTES ON THE RUBBER PARAFFIN METHOD FOR EMBEDDING TISSUES*

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WITH THE ASSISTANCE OF SYDNEY H. KANE, STUDENT

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Search for suitable methods for embedding embryonic chick tissues was one of the many problems encountered in some of our experimental work. We are contributing the following modifications of the rubber paraffin method so that others may benefit by its simplification.

We have experimented with ordinary crude rubber which has the disadvantage of requiring the precaution of working under a hood. In looking around for a more suitable preparation, we were fortunate in coming upon a type of pale crepe rubber† which is much more satisfactory to use. This is pure, virgin

* Received for publication, March 4th, 1938.

† This rubber can be purchased from the Quaker City Rubber Company in Philadelphia, Pa.

rubber and unprocessed and has the advantage of coming in thin layers which are easily separated and cut.

Method. The rubber is peeled layer by layer until the last thin sheet is reached. A piece approximately 20 grams in weight is cut as fine as shredded cocoanut. Then 20 grams of these fine shreds are weighed out and added to 160 grams of melted paraffin in a liter beaker. Either the 53 or 58 degree melting paraffin can be used. The beaker with its contents is now put in a 78 degree Centigrade oven. There are two ways of adding the shredded rubber: (1) the entire amount can be stirred in slowly at one time, (2) 5 grams can be added every 12 or 24 hours with frequent stirring. We have found the latter best in our work, for the rubber dissolves more rapidly and without the appearance of lumps, so that we do not have to filter.

The use of this pale, crepe rubber eliminates the need of working under a hood. The end product is practically a colorless, homogeneous mixture instead of the usual dirty brown. We do not recommend melting the rubber separately and then adding it to the melted paraffin, because the rubber turns a dark brown, making the paraffin dark and leaving an undissolved sediment which has to be filtered out.

This rubber paraffin mixture is kept in the 78 degree Centigrade oven for four days or until the lumps of rubber are gone and the mixture is homogeneous. It is essential to stir the rubber from time to time. It can then be transferred to a 55 or 60 degree Centigrade oven, depending on the melting point of the paraffin used. This is the stock mixture and it can be kept at the back of the oven to be used when needed. One of us finds it more convenient to dilute the entire stock mixture for embedding: another prefers to keep the stock mixture at the back of the oven to make up small quantities at a time. It is safe to keep the stock mixture in the oven as long as the rubber does not separate out from the paraffin.

For embedding, 12½ grams of the stock mixture are added to 500 grams of either 53 or 58 degree Centigrade melting paraffin and 5 grams of bees wax or of bayberry wax. This is placed in a 60 degree Centigrade oven and mixed from time to time. If the lower degree paraffin is used a lower temperature oven can be used. The diluted preparation can be taken out of the oven and kept in the hardened state until 24 hours before use, then it can be put in the oven and melted.* The stock rubber paraffin mixture is difficult to melt once it has hardened. The dilute mixture contains roughly 0.25 per cent melted rubber which seems to be the amount necessary to make a fairly firm block. However, it is advisable to experiment a little in order to find out the optimum hardness desired for each type of tissue, which can be controlled by increasing or reducing the amount of the stock rubber paraffin in the diluted mixture.

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* Some batches have been kept in the hardened state for two years without separation of rubber or paraffin and without deterioration of cutting qualities.

This modified method makes an excellent block for paraffin sectioning in the summer, for extreme chilling is unnecessary. Its use is very satisfactory for general methods, particularly for eye sections, which are notoriously difficult. Rubber paraffin infiltration and embedding does not exclude the use of any of the standard technics of dehydration and clearing (dioxan, alcohol, xylol, etc.).

In conclusion, the advantages of the use of rubber paraffin for embedding are: (1) better infiltration of dense tissues, especially embryonic organs, (2) superior cutting qualities of blocks and (3) desirability for use in warm weather.

ADDENDUM

When it is necessary to cut and mount serial sections in extremely hot weather above 92 degrees Fahrenheit and 53 degrees Centigrade melting paraffin is used, it is helpful to place the paper holding the rubber paraffin section ribbons on a thin sheet of metal (tin, brass, copper) of similar size, which rests on a trayful of ice cubes. This chilling is sufficient to keep the ribbons from sticking to the paper while some of the serial sections are being mounted on slides.

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DOUBLE IMBEDDING METHOD FOR RUBBER PARAFFIN*

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This method was instituted for preparing sections of blood vessels, endocrine glands, and gastro-intestinal tract or any large sections. It is superior to paraffin imbedding for any tissue which varies in density. For trichrome staining where

* Received for publication July 8th, 1938.

five-micron sections are essential this method is invaluable since it permits cutting the entire specimen without fraying.

The material found most suitable for this purpose was a commercial preparation of natural rubber latex called Heveatex which is 37 per cent rubber in an aqueous solution with a small amount of ammonium hydroxide as a preservative. There are a number of other rubber latex preparations, but these contain preservatives such as sodium hydroxide which are more difficult to remove and may interfere with Nissl stains. The Heveatex is spread in a thin film on a glass plate and allowed to air dry for 24 hours or until it is a light brown translucent film, then it is pulled off the plate and cut into small pieces with a scissors; it is now ready to put into the paraffin.

To 100 grams of paraffin add 2 grams of rubber and 0.5 gram of beeswax. Heat on an electric plate at 105° C for 16 hours with occasional stirring. It should then form a homogeneous mixture and be ready for use. Large amounts may be made and kept on hand, but care must be taken in reheating as too high temperatures vulcanize the rubber and it precipitates out of the paraffin.

We have found this ratio of rubber to paraffin most successful for general work. If larger percentages of rubber are used the blocks must be thoroughly iced in an electric refrigerator and not brought out until ready to cut. In extreme hot weather even the 2 per cent mixture may require ice box icing.

The rubber paraffin method gives the best results when used in conjunction with double imbedding. The following method we have found satisfactory for visceral as well as neural tissue:

1. 10% formalin.....24 hours
2. 80% alcohol.....24 hours
3. 95% alcohol.....24 hours
4. Absolute alcohol.....24 hours
5. Absolute alcohol and ether.....24 hours
6. 4% celloidin.....24 hours
7. Absolute alcohol and ether..... 1 hour
8. Xylol for 15 minutes to ½ hour or until transparent.
9. Then into 56–58°C. rubber paraffin in oven three changes for 1½ to 3 hours.
10. Imbed in rubber paraffin the same as paraffin.

For laboratories which handle only a small amount of nervous tissue, this is most useful, since it gives the same picture as celloidin sections as far as shrinkage is concerned.

THE USE OF DIOXAN IN HISTOLOGIC TECHNIQUE*

THELMA GARVIN

From the Department of Pathology of the Nelson Morris Institute, Michael Reese Hospital, Chicago

Dioxan was first used in histological work by Graupner and Weisberger in 1931.[1] These authors subsequently elaborated upon their original work.[2] With this introduction, dioxan was first brought into this laboratory as a possible reagent for the preparation of quick paraffin sections in those instances in which it was either expedient or necessary to have the microscopic diagnosis in as short a time as possible. After considerable experimentation, testing all factors individually and combined, a basic method for running rapid paraffin sections was evolved. The object always in mind was to obtain a final product which was in all respects the equivalent of sections run through much slower, a section for a permanent file and not to be discarded immediately after diagnosis.

Several studies on the use of dioxan have been made by various authors during the last two years,[3, 4, 5, 6, 7] with variation or modification of its use in different circumstances. The method worked out in this laboratory differs from those published in certain details. It is a well known fact that different tissues often require different treatment. By the introduction of 95 per cent alcohol into the procedure, better fixation of all types of tissue is obtained.

The method used is essentially as follows:

1. Rapid fixation in a solution of 10 cc. of 40 per cent commercial formalin in 90 cc. of distilled water. Small fragments from a curettage specimen or biopsy are fixed by heating over a low gas flame until the vapors

* Received for Publication April 28, 1938.
Aided by a grant from the A. B. Kuppenheimer Fund.

arise. Larger sections, not more than 2 mm. thick, are fixed by heating 1–2 hours in an incubator at 60–65°C.

2. 95 per cent alcohol—1 hour.
3. Pure dioxan—2 changes of 1 hour each.
4. Dioxan-paraffin. A 50/50 mixture 1 hour.
5. Paraffin—two changes of 15 minutes to ½ hour each.
6. Embed, cut, mount and stain as for regular paraffin method.

The total interval required from the time the tissue is received until the slide is ready for diagnosis is 5–8 hours. Steps 2–5 inclusive are carried out in an incubator at 60–65° C. Dioxan does not cause undue shrinkage or hardening of the tissue so that sections brought in late in the day can be left at any dioxan step over night.

By trial and comparison we found that by placing the sections in 95 per cent alcohol before putting them into dioxan, better fixation of all types of tissue was obtained than when alcohol was not used, although with very small sections, and if greater speed is imperative, this step may be omitted without greatly impairing the result. The paraffin-dioxan mixture may also be omitted and the sections placed directly into paraffin with two changes of ¾ to 1 hour each. However better infiltration of paraffin, especially with the larger blocks, is obtained by using the mixture.

On sections carried through by this method, all the stains which are ordinarily applied to paraffin sections, such as hematoxylin and eosin, Giemsa, Van Gieson, Gram-Weigert, Ziehl-Neelsen and others, have given results not noticeably different from those obtained on sections which were run by the older ethyl alcohol-chloroform or xylol method. Sections can be cut as thin as 2–3 micra with no more than the usual difficulty, and photomicrographs have been made with good results.

In extending the use of dioxan, we find that by substituting it for the higher percentage alcohols and chloroform or xylol of the older paraffin embedding methods, when working with decalcified material, less brittleness and hardness of the calcareous and cartilaginous substances occur, resulting in greater ease in sectioning.

The question of the toxicity of dioxan is of considerable importance in accepting it for use as a general laboratory reagent. This question is the more pertinent in view of its relationship to ethylene glycol, which substance was found to be the toxic factor in recent deaths attributed to preparations of sulfanilamide.[8] That it is less toxic than ethylene glycol might be surmised by comparison of the chemical formulas.

Ethylene glycol Dioxan (diethylene dioxide)

The original compound with free hydroxyl groups is much more reactive chemically than the ether derivative. Von Oettingen and Jirouch[9] report the minimum lethal dose of glycol to be 2.5 cc./kgm. mouse and that of dioxan above 10 cc./kgm. mouse. Barber in a report from Guy's Hospital, London,[10] reviews five fatal cases of acute dioxan poisoning. He, with other investigators[11, 12, 13] makes the observation that there is little danger of chronic poisoning with fatal results from the use of dioxan provided that it is handled with the usual precautions with which all volatile laboratory reagents should be handled; namely, in well closed containers in well ventilated rooms.

The method for rapid paraffin embedding of sections using dioxan has been used in our laboratories for a period exceeding two years with universally good results. The method, with modifications and suggestions presented, is only an indication of the possibilities of the use of dioxan and offered as concrete evidence of some of the advantages of dioxan over the better known reagents in the tissue laboratory.

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THE BLOOD BANK OF THE GRADUATE HOSPITAL OF THE UNIVERSITY OF PENNSYLVANIA*

P. B. PATTON

From the Graduate Hospital of the University of Pennsylvania, Philadelphia, Pa.

The method is still more or less in an experimental stage but, because it has functioned satisfactorily for the collection, preservation and use of 400,000 cc. of blood, is briefly outlined below.

Blood is collected from *fasting* donors after careful questioning and examination to rule out syphilis, gonorrhea, malaria, inguinal buboes, lymphogranuloma venereum and other diseases.

* Demonstrated before the Technician's Institute held in Philadelphia, April 11–13, 1938, under the direction of John A. Kolmer, M.D., Temple University School of Medicine.

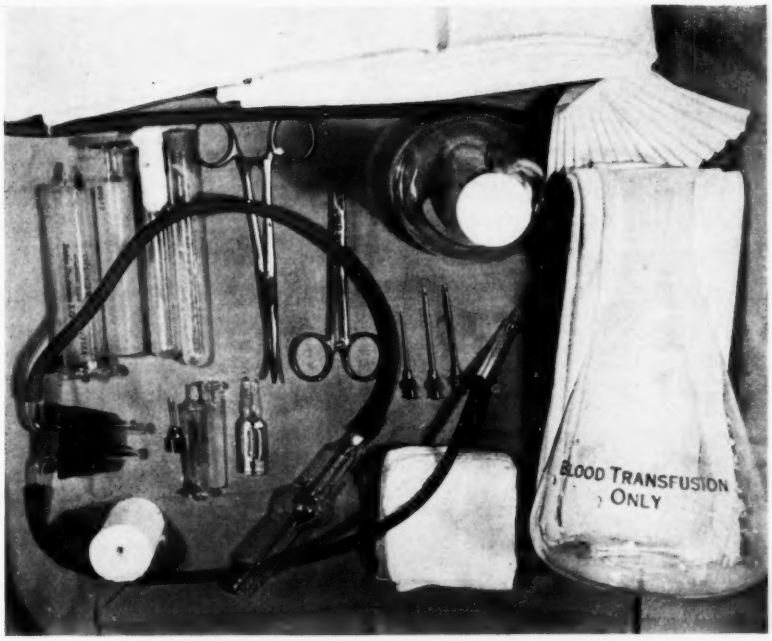

FIG. 1. EQUIPMENT REQUIRED FOR COLLECTING BLOOD

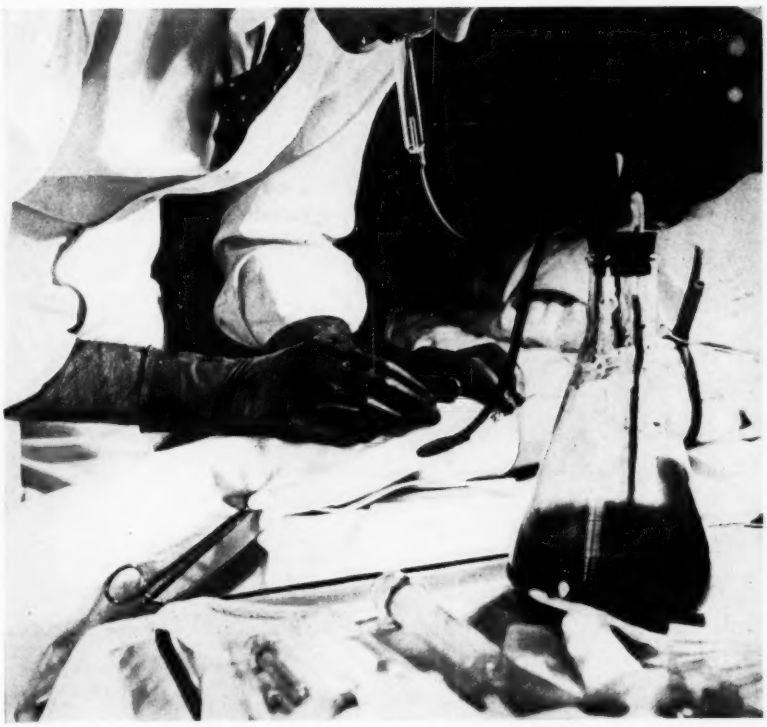

FIG. 2. COLLECTION OF BLOOD FROM DONOR

The blood is drawn into an Erlenmeyer flask, using mouth suction to produce a vacuum. The equipment necessary for the collection of blood is shown in figure 1 and the actual collection in figure 2.

An average of about 500 cc. is collected from each donor and gently mixed with about 70 cc. of 2.5 per cent citrate. Ten

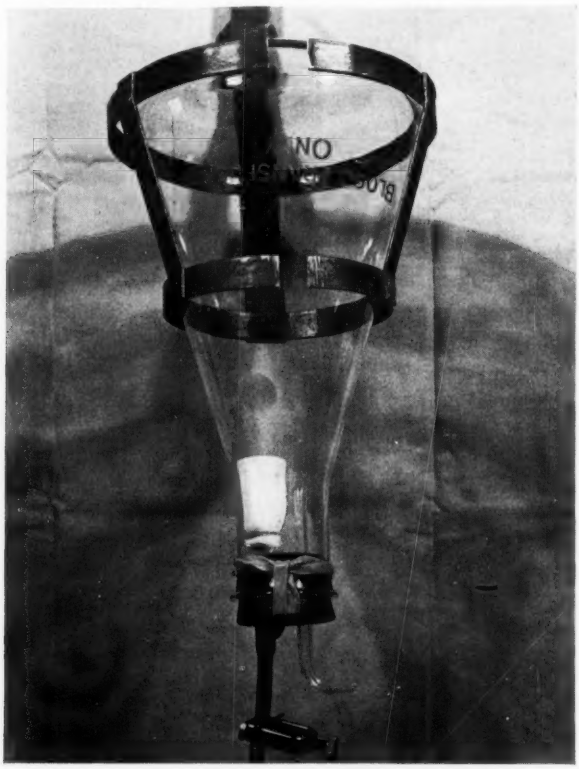

FIG. 3. ADMINISTRATION UNIT IN POSITION (WITHOUT FLUID) TO SHOW ARRANGEMENT OF FILTERS

centimeters of whole blood is placed in a sterile test tube for Wassermann and flocculation tests and an equal amount in an oxalated tube for typing. The serum and cells thus secured are available for compatability tests prior to the use of the blood for transfusion. Preservation is in an electric refrigerator at 4–5 degrees Centigrade for a maximum period of 21 days.

The blood is administered to the recipient from the same flask in which it was collected by means of the simple apparatus shown in figure 3. No attempt is made to raise the temperature of the blood before administration. Artificial heating has increased the incidence of reactions.

The flask is stoppered with a two-holed rubber stopper. An air filter is placed in one hole and in the other a special blood

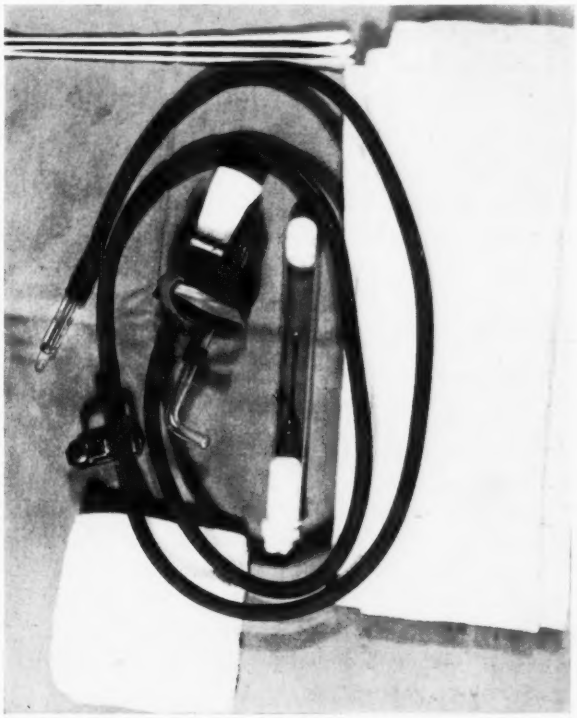

FIG. 4. THE ADMINISTRATION UNIT AS DISPENSED

filter which is merely a piece of glass tubing with the end flared and having vents in the side of the tube. This is covered with a three-layered gauze cap which acts as a filter.

The capillary air filter allows air to bubble through the blood, thus mixing it. Figure 4 shows the administration unit as dispensed.

In administering the blood to the recipient the flask is inverted, as shown in figure 4, the blood entering the vein via the rubber

tubing which carries a glass adapter for the needle, the air filter preventing a vacuum.

The maintenance of clean, sterile equipment is in the hands of the Surgical Supply Room. Emphasis is placed on the avoidance of harsh soaps and the use of *large* amounts of tap water, preferably under pressure, and *large* amounts of distilled water for all cleansing purposes. New rubber tubing is autoclaved 30 minutes in 5 per cent sodium carbonate solution, and then thoroughly rinsed under pressure.

It is important that all equipment be autoclaved and ready for use within two hours from the time the cleansing process is started.

In summary, this system has provided an adequate amount of readily available blood which has been administered easily, without any reactions other than the unavoidable allergic type.

ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

NEW CORNING GLASS WORKS CATALOG

The Corning Glass Works have issued a new and extremely comprehensive catalog of "Pyrex" brand ware (L. P. 18). It contains 128 pages, and carries 2,353 individual items, of which over 700 are listed for the first time. Some of the features of the new catalog are that practically all ground joints, stoppers, and stopcocks listed are now interchangeable; number of pieces per package reduced on many items; and prices are reduced on more than 500 items, including centrifuge tubes, condensers, distilling flasks, ground joints and connections, separatory funnels and stopcocks, tubes for clinical laboratories, as well as some generally used sizes of test tubes, tubing, and rod.